Conventional Scanning Electron Microscopy of Bacteria

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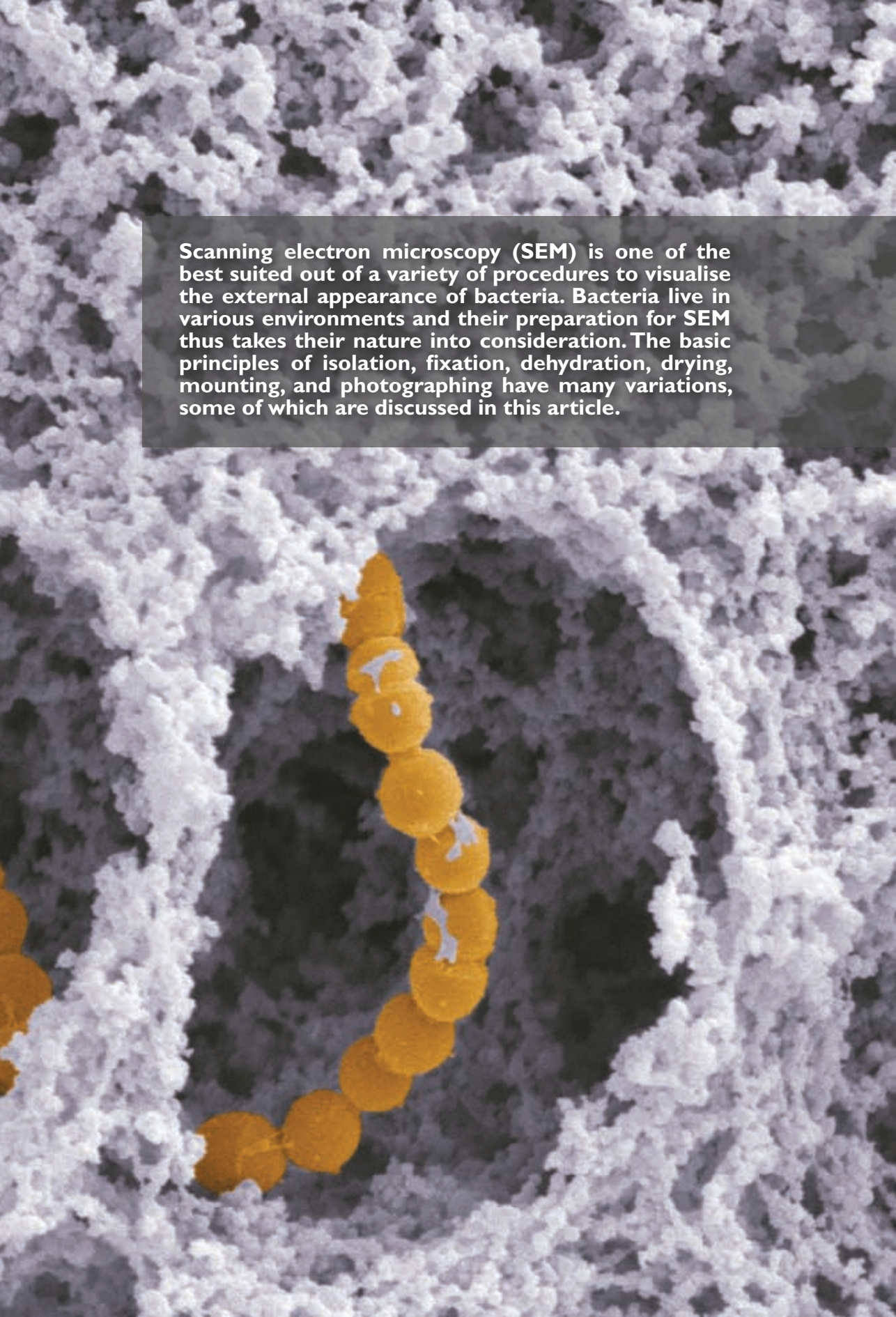


Scanning electron microscopy (SEM) is one of the best suited out of a variety of procedures to visualise the external appearance of bacteria. Bacteria live in various environments and their preparation for SEM thus takes their nature into consideration. The basic principles of isolation, fixation, dehydration, drying, mounting, and photographing have many variations, some of which are discussed in this article.

Bacteria are present everywhere: in the soil, deep in the rocks, in all bodies of water, in the atmosphere, including the clouds, and also on and inside other living organisms.

Introduction

Bacteria are present everywhere: in the soil, deep in the rocks, in all bodies of water, in the atmosphere, including the clouds, and also on and inside other living organisms. Their effects on higher life forms are known for only a limited number of bacterial species. Some are harmful - pathogenic - causing diseases in plants or in animals including humans, and some are useful to humans either as "probiotic" bacteria protecting health (Karpa, 2006), or industrially by participating in the production of various commodities. The effects of most bacteria are not known. A new trend is in progress (Sachs, 2008) not to exterminate harmful bacteria in human and animal environments but to replace them with beneficial ones. Bacteria which are genetically programmed to die after their mission has been accomplished in humans or animals are part of the new trend. These minute microorganisms have far-reaching macroscopic consequences. They are of interest to agriculture because they may harm or improve the production of foods, either animal or plant, and thus affect human civilisation.

Electron microscopy is one of a limited number of techniques suitable to show bacteria in great detail in their natural environment. There are two basic modes, scanning electron microscopy (SEM) and transmission electron microscopy (TEM), and each of them uses specially designed microscopes. SEM is relatively easy to perform and the results are easy to interpret, because the specimens are shown as three-dimensional objects (Figure 1). TEM produces different kinds of images based on the fact that the electron beam passes through the specimen and forms a shadow-like image on a fluorescent screen which is photographed. Several examples of bacteria visualised by TEM are shown to distinguish them from images obtained by SEM.

In negative staining, a dilute suspension of unfixed bacteria is applied on a Formvar film followed by addition of a dilute heavy metal salt solution (e.g., uranyl acetate, ammonium molybdate, or sodium phosphotungstate). When the dried specimen is placed into the path of an electron beam in the TEM, the electrons are absorbed by the heavy metal but they pass through the bacteria and form their images on a fluorescent screen. This procedure is excellent to show bacterial fimbriae, flagella, etc. (see Figure 2).

Embedding bacteria in a resin and staining thin sections of the resulting bloc with heavy metals such as uranyl acetate and lead citrate (Reynolds 1963) reveals the internal structure of the bacteria (Figure 3).

One of the most elaborate TEM techniques consists of rapidly freezing the bacteria, freeze-fracturing them, replicating the newly developed surfaces with platinum and carbon, and examining the replicas. Haggis & Bond (1979) developed a replication procedure for dried specimens which was later used by Kalab (1980) to visualise bacteria in cultured milk products (Figure 4).

Scanning electron microscopy

Like TEM, SEM also consists of different techniques. Conventional SEM is used to visualise bacteria which had been fixed, dehydrated, and dried. Rapidly frozen (cryofixed) bacteria may be examined at very low temperatures (below –120°C) by cryo-SEM. Both techniques are carried out with

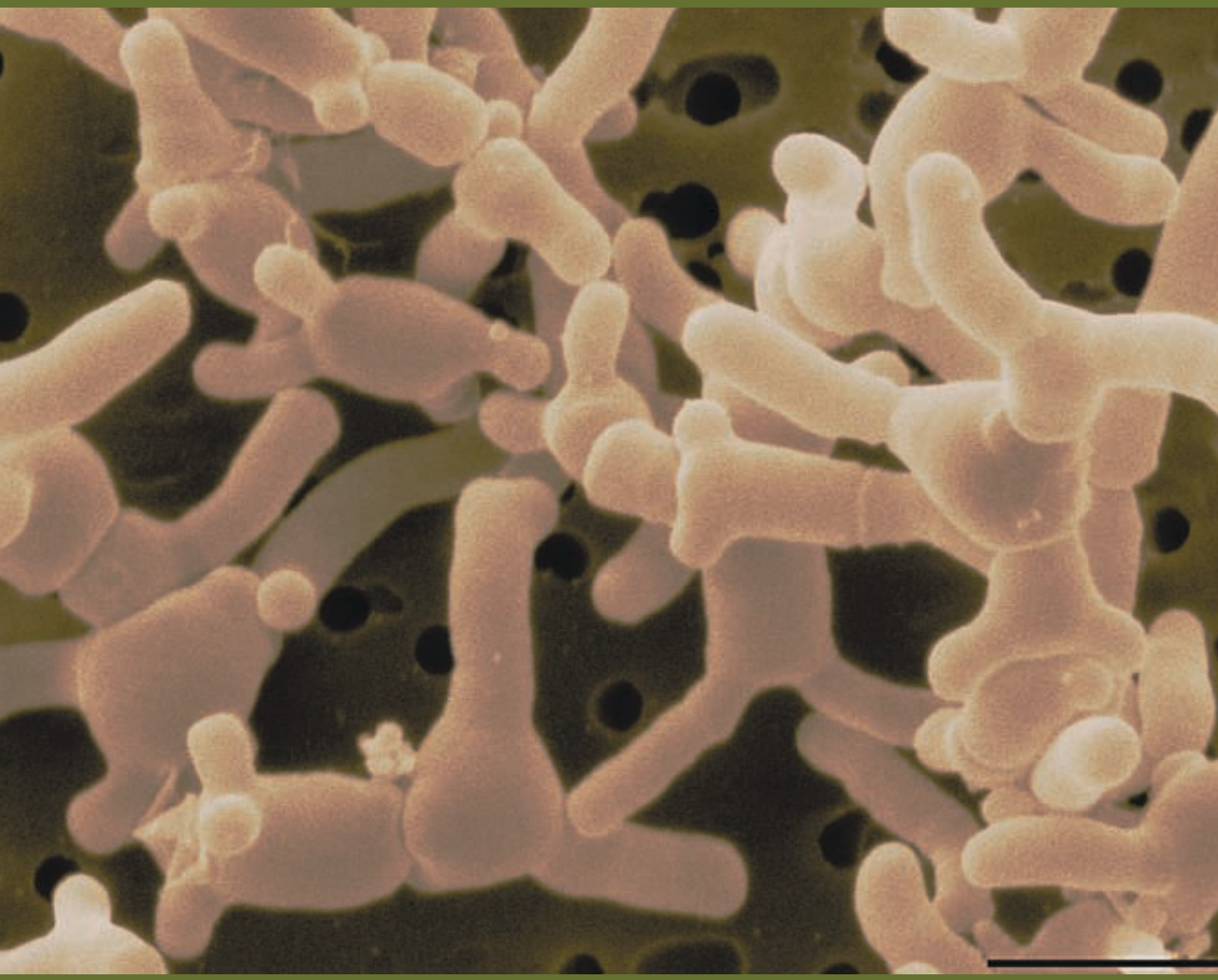


Fig. 1. SEM of a cluster of Bifidobacterium breve bacteria on a bacterial filter. Bifidobacteria are gram-positive, anaerobic, branched rod-shaped probiotic bacteria with a very wide variety of species. Bar: 2 µm.

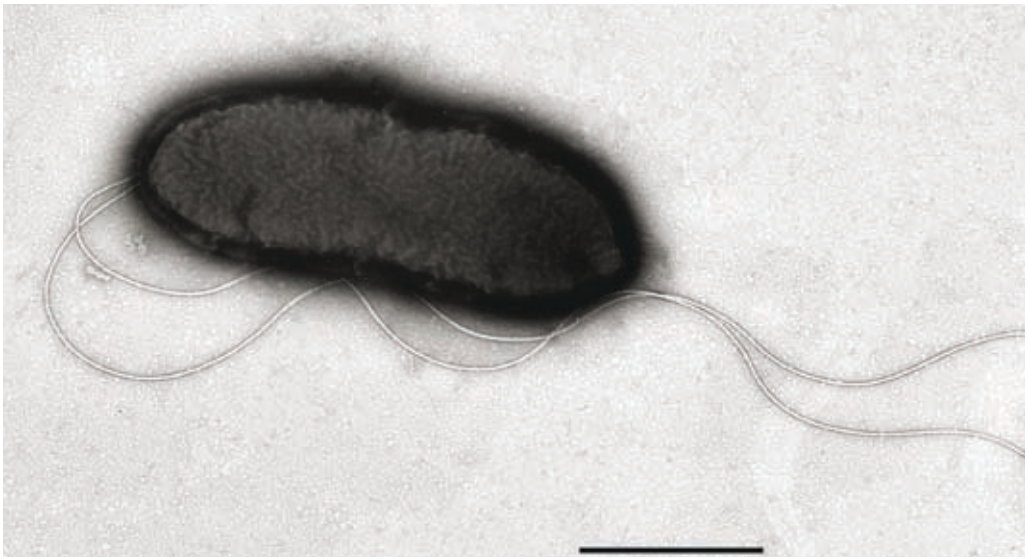


Fig. 2. Negative staining of a Gluconoacetobacter spp. bacterium with phosphotungstic acid reveals the flagella. Bar: 1 µm.

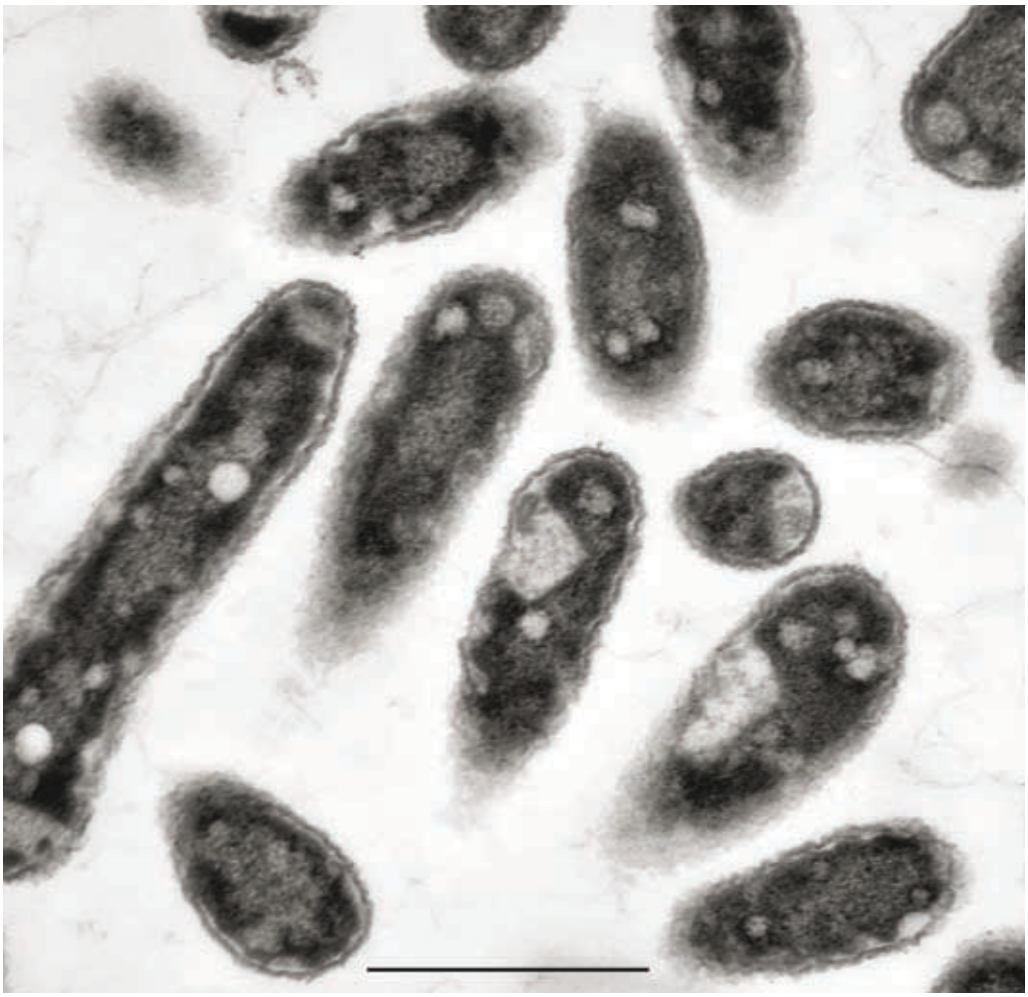


Fig. 3. Thin section of Pseudomonas fluorescens bacteria. P. fluorescens are gram-negative, rod-shaped bacteria with multiple flagella. Bar: 1 µm.

the specimen placed in a high vacuum chamber. There, the specimen must not release any volatile substances. Drying or freezing at a very low temperature meets this requirement. Environmental SEM (ESEM) makes it possible to examine hydrated specimens by not exposing them to high vacuum. A sophisticated technical design keeps the specimen at a temperature several degrees above the freezing point of water in a small area inside the microscope where a low partial pressure of water vapour provides ions at a concentration sufficient to neutralise electrons and thus to prevent charging artifacts (Kalab 1984). Cryo-SEM and ESEM have their own specific features which in some aspects differ from conventional SEM.

Every specimen destined for conventional SEM must be dry.

Each of the electron microscopy modes mentioned has advantages and disadvantages. Conventional SEM is a relatively simple and rapid technique and the images are easy to interpret, but it does not provide as high resolution as TEM. In principle, the preparation for SEM consists of isolating the bacteria or trimming the specimen where they are present, fixing them, dehydrating in ethanol, critical-point drying, mounting on an SEM stub, sputter-coating with gold, and recording images at an appropriate accelerating voltage.

Dehydration and critical-point drying may disturb bacterial flagella. Sputter coating the fixed and dried bacteria with a 20 nm thick layer gold obscures fine structures such as pili and fimbriae. However, such structures may be visualised by TEM using negative staining or shadowing with platinum.

SEM Preparation Procedures

Every specimen destined for conventional SEM must be dry. As living microorganisms, bacteria contain proteins and a high proportion of water in their cells. It is essential to fix them first in order to preserve their structure while they are being further prepared for SEM. To accomplish such steps in reasonable time, the specimens should be relatively thin (<2 mm) and small (only a few mm). Large solid materials which contain bacteria on their surfaces such as contaminated meat, skin, vegetables, composted materials, or agar gel plates with bacterial colonies are first excised and trimmed to approximately 10 mm x 10 mm specimens as thin as possible (1 - 2 mm), and fixed before they are further reduced into smaller (approx. 5 mm x 5 mm) particles.

If the bacteria are inside a specimen such as set-style yogurt, cheese, kefir grains and other materials which may easily be cut, it is advantageous to trim them into prisms 10 to 15 mm long and less than 1.5 mm x 1.5 mm in cross section. Viscous specimens such as stirred yogurt and cream are best encapsulated in agar gel tubes in the form of 10 to 15 mm long columns (Allan-Wojtas & Kaláb, 1984a), whereas heat-sensitive foods such as raw egg yolk may be encapsulated in calcium alginate gel tubes (Veliky & Kaláb, 1990). Bacterial colonies on agar gel plates are excised with up to 1 mm of a clear agar gel rim. If possible, the bacteria destined for SEM should be grown on thin plates (<2 mm) to make the excision easier.

Solid specimens are fixed in a buffered (0.1 M, pH 6.5-7.0) fixative such as 2-3% glutaraldehyde for periods ranging from 5 min to 24 h (Glauert, 1975a). If specimen constituents not based on protein such as lipids (fat globules in food products) or polysaccharides (mucus in the intestines) are also present, postfixation using osmium tetroxide (OsO_4) or Ruthenium Red, respectively, is used. Although double bonds (–C=C–) in unsaturated fatty acids react with osmium tetroxide, osmium is easily removed by subsequent spontaneous hydrolysis. It results in the formation of a diol and the release of free osmium trioxide (OsO_3) (Greyer

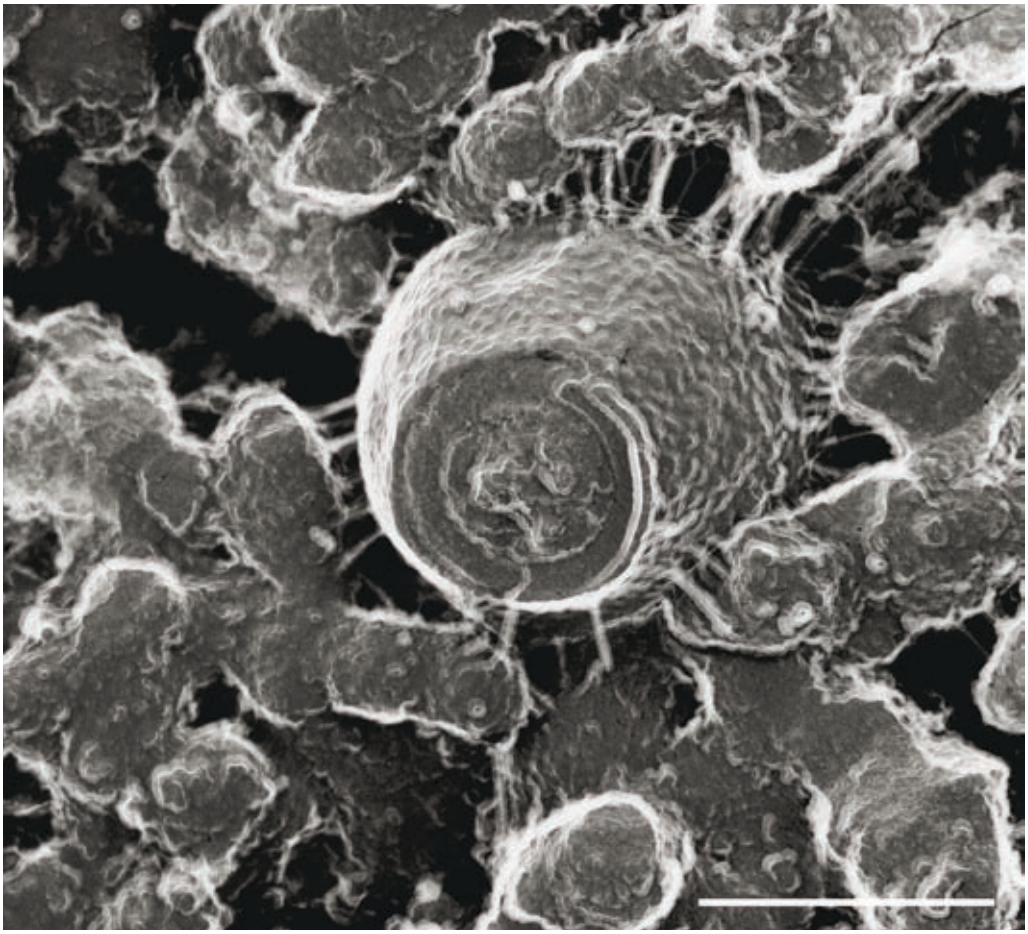


Fig. 4. Replica of a lactic acid bacterium in Cottage cheese. The specimen had been fixed, dehydrated in ethanol, freeze-fractured, thawed, critical-point dried, and replicated with platinum and carbon. Bar: 1 µm.

1977). No hydrolysis takes places, however, if a heterocyclic base such as pyridine or imidazole is present in the fixative (Angermüller & Fahimi, 1982). Imidazole-buffered OsO_4 preserves the fat and oil particles for SEM in specimens such as cheese, yogurt (Allan-Wojtas & Kaláb, 1984b), hard-boiled eggs, and comminuted meat products.

The need to fix intestinal mucus to visualise bacteria attached to the intestinal walls was encountered by Allan-Wojtas *et al.* (1997). Conventional preparation of intestinal specimens, however, may fail to retain the bacteria because they are washed off from the intestines along with the mucus covering them. The mucus may be fixed using Ruthenium Red or Alcian Blue (Erdos, 1986) added to the glutaraldehyde fixative. Our own experiments on chicken intestines (not yet published) indicate that the intestinal wall specimens need to be freeze-fractured and viewed sideways in order to see the bacteria which are below the mucus.

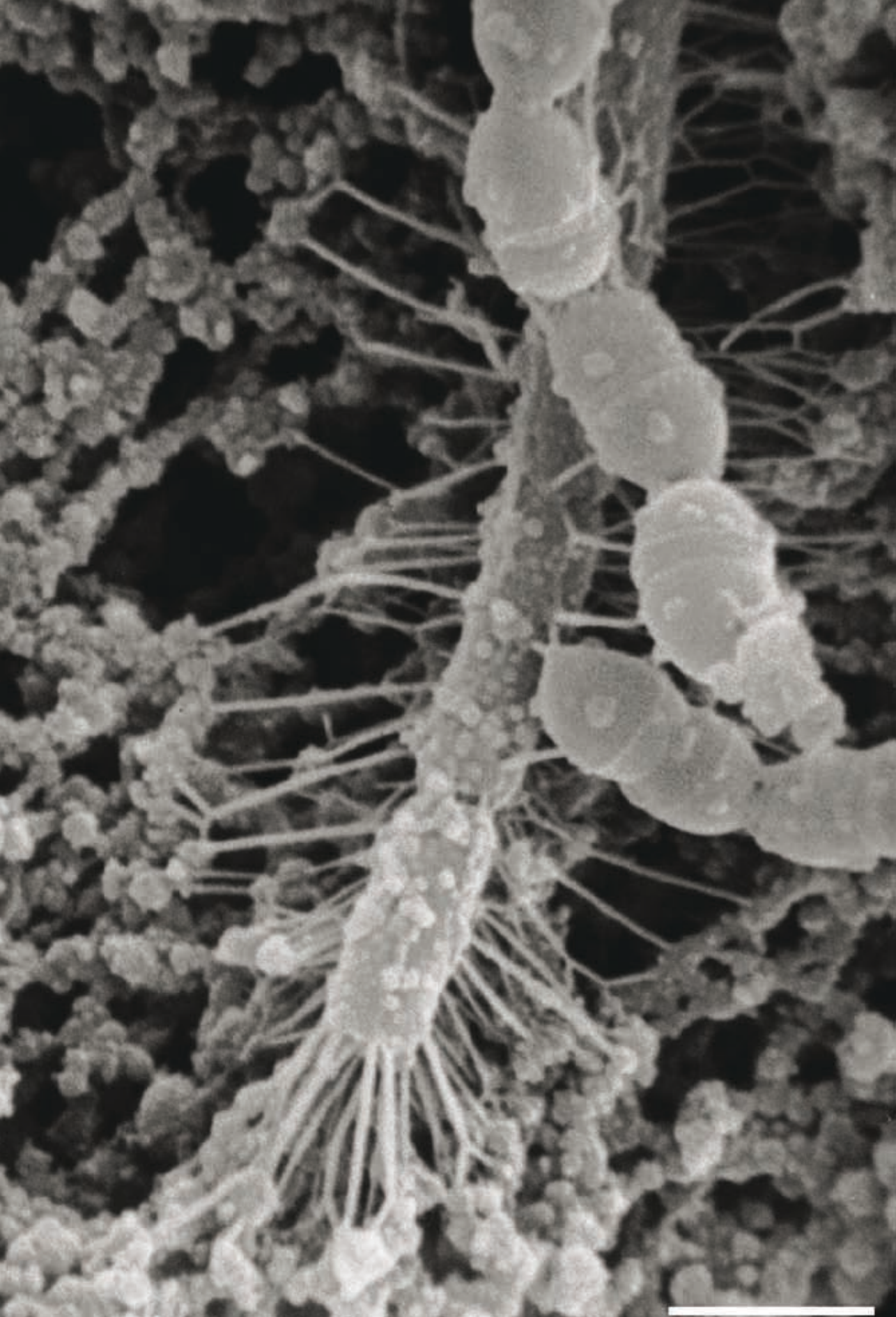
Fixed specimens are washed with the corresponding buffer and dehydrated in a graded ethanol series. Foods may be considerably denser than the sources from which they had been produced. For example, cheeses are very dense, particularly low-fat cheeses and processed cheeses, ham is considerably denser than muscle. Such specimens thus require longer periods for both fixation and dehydration. The fixative and dehydrating solutions must penetrate the entire specimens to make the SEM examination

relevant. There is a relatively easy test to check osmium tetroxide fixation of yogurt and cheese. Prisms, 1 mm x 1 mm in cross section, initially fixed in a glutaraldehyde fixative for a minimum of 2 h and washed with a buffer are placed in a 1% or 2% OsO_4 fixative and allowed to stand at 6°C or 20°C for 2 to 24 h. Cutting off a small part of each prism after 2, 12, and 24 h reveals how deep into the interior of each food sample OsO_4 has penetrated - that area is markedly darker. Yogurt is usually completely fixed in 2 h, whereas only a thin superficial layer in the cheese prism will turn dark.

Specimens with bacteria present inside, such as the foods mentioned above, need to be opened for viewing. Dry-fracturing breaks the dried specimen at the weakest points and produces a coarse topography of the new surface. Bacteria are not broken by this process and remain intact. Freeze-fracturing produces smooth fracture planes and the new surfaces are flat, making it possible to evaluate the shapes and dimensions of other features such as bacteria, yeasts, starch granules, fat globules, and void spaces. Naturally, bacteria present in the area of the fracture also become fractured.

Freeze-fracturing is done after the fixed specimens have been completely dehydrated in absolute ethanol. The specimens are frozen in liquid nitrogen although it is an inefficient cryogen. It is a liquid at the temperature of its boiling point. When a specimen is immersed into it, an insulating layer of gas surrounds the specimen and decelerates the freezing rate. There are several procedures to improve the freezing rate (Moor, 1973). Better cryogens such as Freons are environmentally harmful and their use has been discontinued. However, freezing in liquid nitrogen is not detrimental to specimens impregnated with ethanol since this solvent does not produce ice crystals when frozen. Thus, there is no risk of developing freezing artifacts (Kalab, 1984). The frozen specimen prisms immersed in liquid nitrogen are fractured using a pair of insulated tweezers and an insulated scalpel (Figure 5). The fragments are picked up one by one and thawed in absolute ethanol at ambient temperature and

Fig. 5. Insulated tweezers (top) and a scalpel (bottom) used to freeze-fracture specimens in preparation for SEM. Black adhesive rubber foam insulation was cut to size and attached to the tools shown.



are subsequently critical-point dried (CPD). The fracture planes are shiny under reflected light and they should face up when the fragments are mounted on aluminium SEM stubs. Double-sided sticky tape on the face of an SEM stub facilitates their positioning. Minute droplets of a conductive silver cement at the proper viscosity are then carefully painted on the side walls of the fragments to attach them to the tape. A cement too thick may not properly adhere to the specimen whereas a cement too thin may soak into the specimen and ruin it. The fragments acquire electrically conductive surfaces after they are sputter-coated with a 20 nm layer of gold. They are then ready to be examined by SEM.

In the past century, so-called "ropy" bacteria could be seen in some dairy products. They produce large amounts of exocellular polysaccharides (Brooker, 1979) called "capsules" which are assumed to protect the bacteria from phagocytosis. These polysaccharides increase the viscosity of the cultured milk product such as yogurt, where they reduce syneresis (separation of the liquid phase from gelled milk proteins) and make it possible to decrease or completely eliminate the use of thickening agents. The trends to use or not to use ropy bacteria change depending on the cost of milk solids and stabilisers. The mouthfeel of such yogurts is somewhat different from yogurts made with non-ropy cultures. In the micrographs, the bacteria appear to be attached to the protein matrix through numerous filaments (Figure 6). They may also be seen in the replica shown in Figure 4. It was erroneously believed in the dairy industry several decades ago that the bacteria attach themselves to the protein matrix in order not to be washed out with the whey. In fact, glutaraldehyde does not fix the polysaccharides surrounding the bacteria (capsules) and they shrink into filaments during dehydration and critical-point drying. The filaments are thus artifacts which originate from the bacterial capsules. Less dense capsules explain the presence of "void spaces," i.e., empty spaces surrounding bacterial chains in some yogurts (Figure 7) – they keep the casein particles at a distance away from the bacteria. When the casein coagulates, the bacteria appear positioned in the middle of an empty space.

Bacteria may be captured on the filters either in live or fixed forms.

Bacteria in suspensions may require preliminary cleaning, preferably by filtering off coarser particles. There is a wide variety of filters which differ by pore diameters suitable for this purpose. Purified bacteria may be captured for SEM on filters, 13 mm in diameter (to fit the SEM metal stubs), which have small (0.2 to 1 µm) pores. Polycarbonate "nuclepore" filters, which have relatively smooth surfaces, resistant to ethanol and liquid carbon dioxide (but not to acetone) are suitable for alcohol dehydration and critical-point drying. Bacteria may be captured on the filters either in live or fixed forms. This kind of filter has one side shiny (very smooth) and the other matte (somewhat coarse) (www.magma.ca/~pavel/science/BactFilters.htm). The latter side appears to hold the bacteria better during subsequent preparatory steps. Live bacteria adhere better than bacteria which have been fixed. A preliminary treatment of the filters with a 0.1% solution of polylysine hydrobromide facilitates adherence of the bacteria to the filters (Weiss 1984). A filter holder with a fine stainless steel mesh as part of the Millipore Swinney Stainless, 13 mm filter (Cat. No. XX30 012 00) filtration syringe) (www.millipore.com/userguides/tech1/p30970) has proven to be very useful when mounted on a vacuum flask using a short flexible tubing (Figure 8). The flask is attached to a vacuum line through a cotton wool filter and a valve. Bacterial suspensions are applied through a Pasteur pipette onto an

Fig. 6. Yogurt made with Lactobacillus bulgaricus (ropy) and Streptococcus thermophilus (non-ropy) bacteria. "Ropy" bacteria appear to be attached to the casein matrix through fine filaments. Bar: 2 µm.

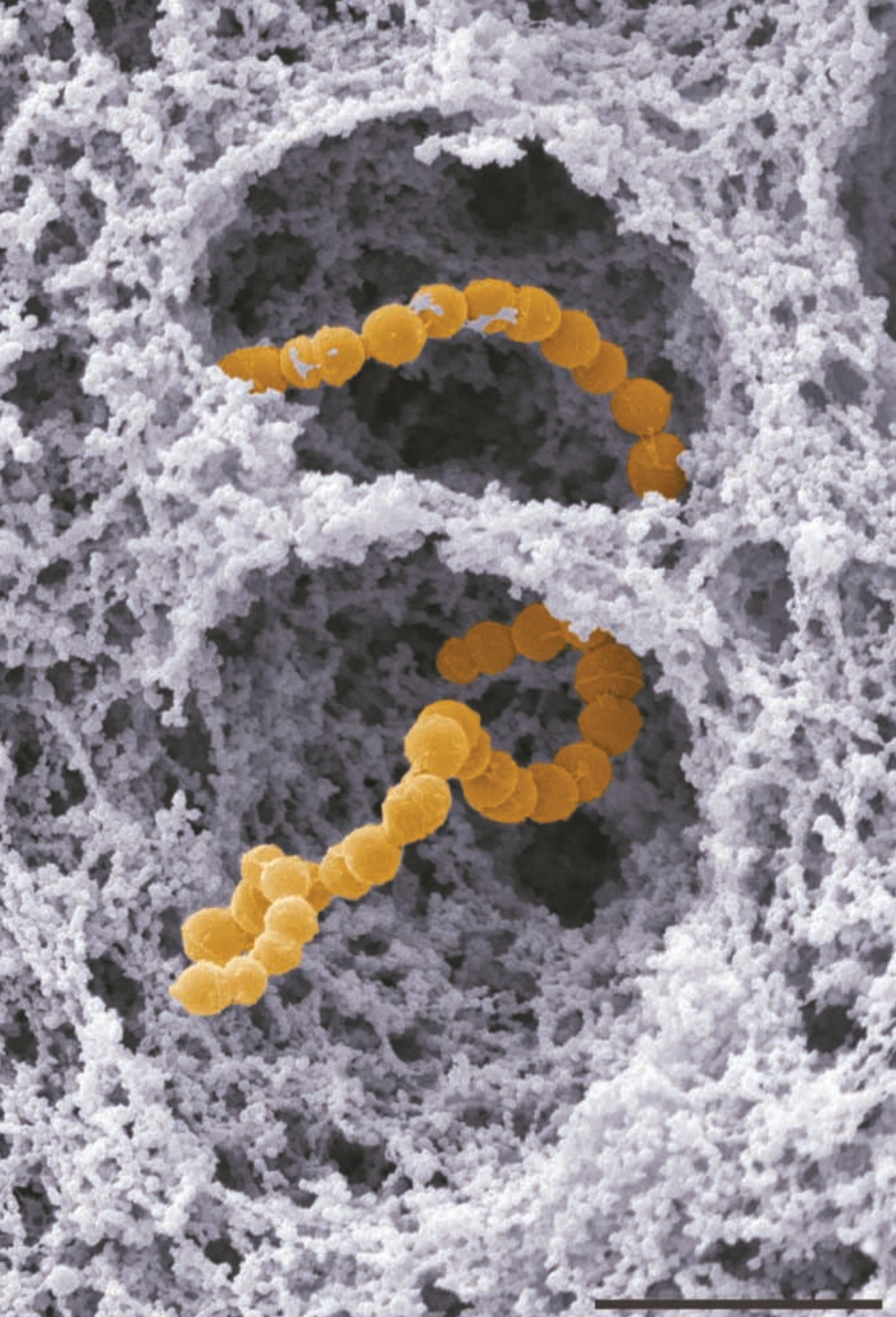


Fig. 8. Depositing bacteria from a suspension onto a nuclepore filter using a Pasteur pipette. The tilted filter holder and the suction distribute the bacteria to form a layer increasing in thickness from thin to thick from the top to the bottom along the slope.

inclined filter. A greater volume of the suspension is filtered through the lower part of the filter, which means that the bacterial layer is thinnest at the top part and is thickest at the lowest part of the filter. Thus, there develops an area with the most desirable density of the bacteria on the filter. The bacteria change the reflectivity of the filter surface. With some experience, the laboratory worker will always achieve the proper density of the bacteria on some part of the filter. The application of vacuum is stopped as soon as there is no visible liquid on the filter but the filter (and the bacteria) must still be wet when transferred either into a fixative or, if the bacteria have already been fixed, into 20% ethanol for dehydration.

Fixation and/or dehydration are best performed in capped wide-neck vials to accommodate the filters. The specimens are dehydrated through a graded ethanol series (20 40, 60, 80, 95, 100, 100, 100% ethanol) on a slowly moving inclined rotary table. Short, 5-10 min, intervals are sufficient to dehydrate several filters in a vial (Glauert 1975b). Finally, the filters are critical-point dried. Multiple specimen holders may be used if needed. With only a few individual samples, fixation and dehydration of the bacteria captured on polycarbonate filters may be carried out in the filter holder. The filter must remain wet all the time. It may be transferred very briefly onto a piece of filter paper to drain residual absolute ethanol before critical-point drying (D. R. McCoy, personal communication). The inlet of liquid carbon dioxide into the pressurized cell is shielded to reduce the risk that the bacteria on the filter will be washed away.

Unlike solid specimens, porous filters must not be mounted on double-sided sticky tape attached to

Fig. 7. Void space caused by Lactobacillus bulgaricus bacteria (orange colour) in yogurt. The void space is explained first by the formation of exocellular polysaccharides (capsules) by the bacteria and then by their removal while the yogurt specimen was prepared for SEM. The thin polysaccharide gel prevented casein micelles in milk from getting into contact with the bacteria. Thus the micelles gelled at a distance from the bacteria. The polysaccharide gel was not thick enough to shrink into the form of filaments like in the case of "ropy" bacteria. Bar: 5 μm.

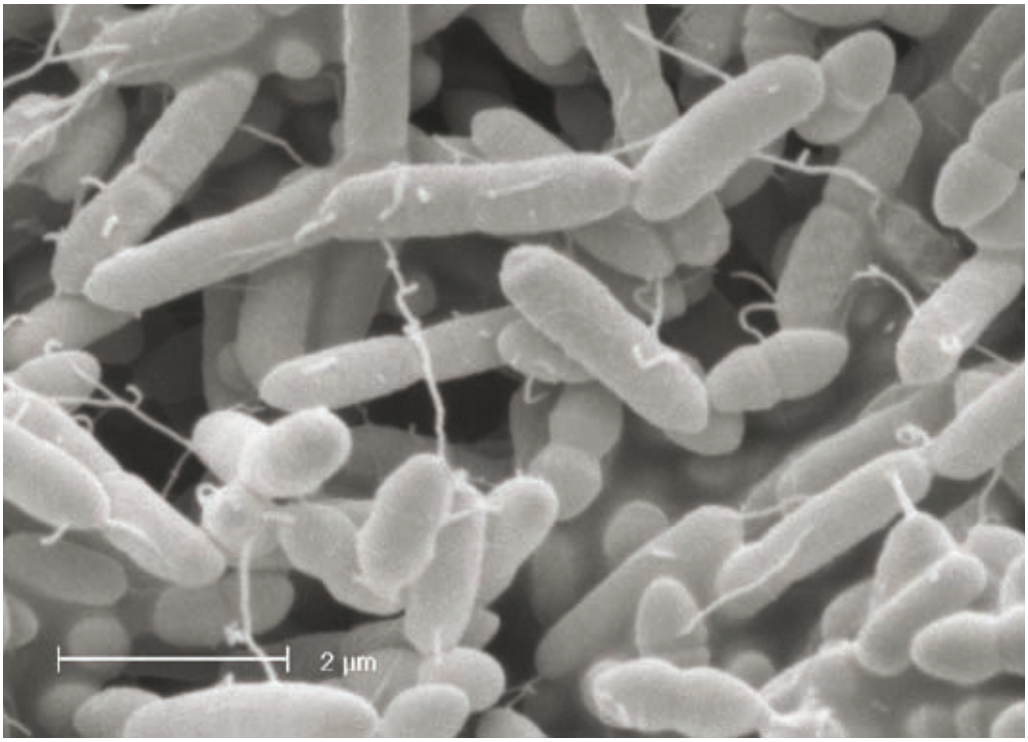


Fig. 9. Staphylococcus griseus bacteria on a filter attached to a double-sided sticky tape on an SEM stub through its entire area. During 2 weeks after mounting, the sticky substances oozed up through the filter pores and distorted the bacteria. The area in the lower right corner is damaged compared to the opposite upper left corner. Bar: 2 µm.

the SEM aluminium stubs if SEM cannot be done until several days later. The adhesive of the tape has been found to slowly penetrate through the filter pores and to adversely affect the bacteria (Figure 9). The filter becomes completely useless (www.magma.ca/~scimat/Sticky_tape.htm) after one or two weeks of storage. It is preferable to attach the filters to bare metal stubs using several 2 mm x 2 mm squares of the double sided sticky tape placed at the edges of the SEM stub. The connections may be strengthened by several minute droplets of a conductive silver cement placed at the filter edges. Filters mounted in this way will not deteriorate in storage for several weeks.

A relatively new method to isolate certain bacteria from their mixtures in suspensions is based on the use of microscopic magnetic beads. Some brands consist of magnetic polystyrene beads coated with antibodies against specific bacterial antigens such as those in toxigenic Escherichia coli and Salmonella typhimurium. When these minute beads are released into a medium which contains a variety of bacterial strains, targeted bacteria will adhere to the coated beads. If this reaction is carried out in a glass beaker or a test tube and a magnet approaches the container, the beads with the bacteria attached concentrate at the glass wall at the magnet. It is possible to pull the beads with the bacteria out of the container by moving the magnet upwards. A small volume (several microlitres or less) depending on the concentration of the beads in the isolate captured on a nuclepore filter may be prepared for SEM in the same way as bacterial suspensions (Figure 10).

There is a growing interest in bacterial biofilms (Greco *et al*., 2007). They form on various surfaces and may be susceptible to disturbances before they become resistant to them depending on the stage of their development. In some cases, the material on which they develop may be cut into smaller pieces.

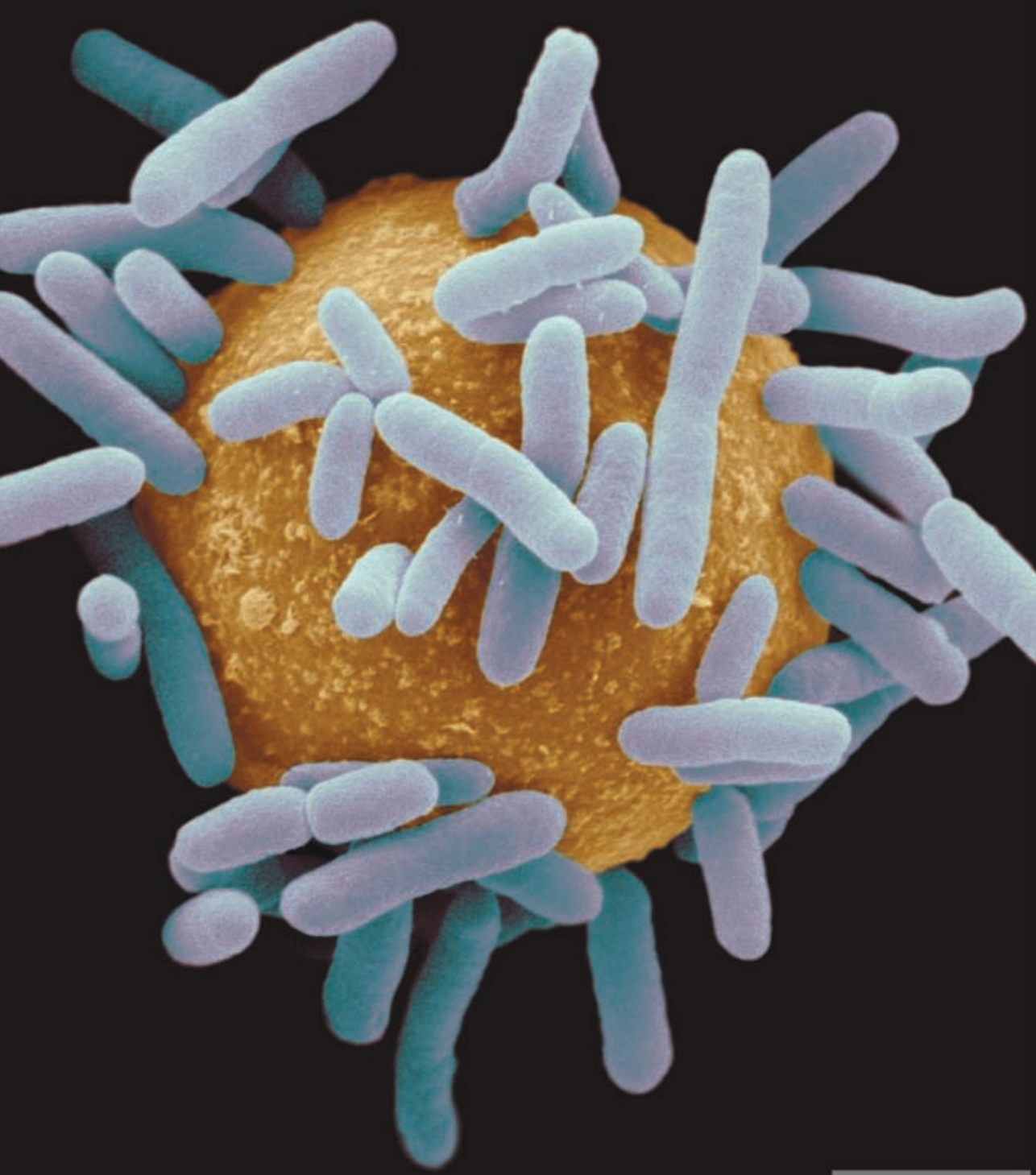


Fig. 10. Escherichia coli bacteria attached to a polystyrene magnetic bead that had been coated with an E. coli antigen. Bar: 2 µm.

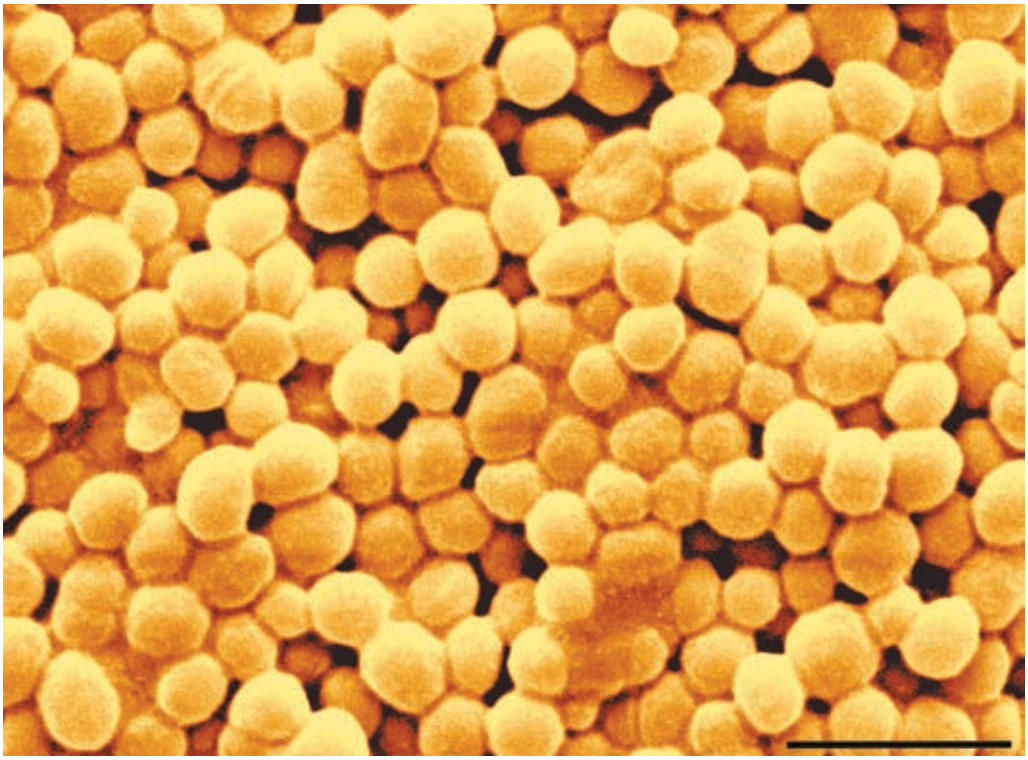


Fig. 11. Staphylococcus epidermidis biofilm on the plastic wall of a transfusion bag. Small pieces of the wall were exposed to the bacteria and then cut into approx. 10 mm x 10 mm squares, fixed with osmium tetroxide vapour, and subsequently allowed to dry in air in a fume hood. The dried biofilm was sputter coated with gold. S. epidermidis gram-positive cocci are usually non-pathogenic but cause infections in people with compromised immune system. Their biofilms adhere to the surfaces of medical prostheses. Bar: 5 µm.

Before information in greater detail can be obtained about their resistance, it is advisable to fix them in OsO_4 vapour in a small air-tight wet chamber. Several drops of a 2% OsO_4 solution placed in the vicinity of a specimen 10 mm x 10 mm for an hour or two may be sufficient to fix the biofilm on it. The specimen may then be dried in air (Figure 11). Had fixation with OsO_4 vapour been omitted, the subsequent air-drying would severely distort the bacterial cells. An alternative treatment is to very carefully dehydrate the biofilm in a graded acetone series using a Pasteur pipette to gradually increase the acetone concentration to 100%. Finally, most acetone is drained off and the specimen is allowed to dry in air. This kind of drying was used with biological tissues before the introduction of critical-point drying. Compared to ethanol, acetone is a strong lipophilic solvent and may have detrimental effects on lipid-containing specimens. Dehydration in ethanol followed by CPD would be a test to learn how the biofilm is resistant to this kind of handling. Shielding the specimens from the flow of liquid carbon dioxide in the pressurised cell is strongly recommended. These preparatory procedures may also be tried with sensitive bacterial colonies on thin agar gel plates.

Bacteria live and form biofilms on metal surfaces where foods are processed. Cotton swabs are used to capture and culture them in diagnostic media for identification. Viewing the bacteria as they are dispersed on the surfaces cannot be done directly because of large dimensions of such surfaces. However, it is possible to obtain their replicas and examine these by SEM. Cellulose acetate strips softened with acetone are pressed against the contaminated hard surfaces and then allowed to dry. The resulting replicas are peeled off and trimmed, mounted on SEM stubs, and gold-coated (Figure 12) (www.magma.ca/~scimat/Replication.htm). The

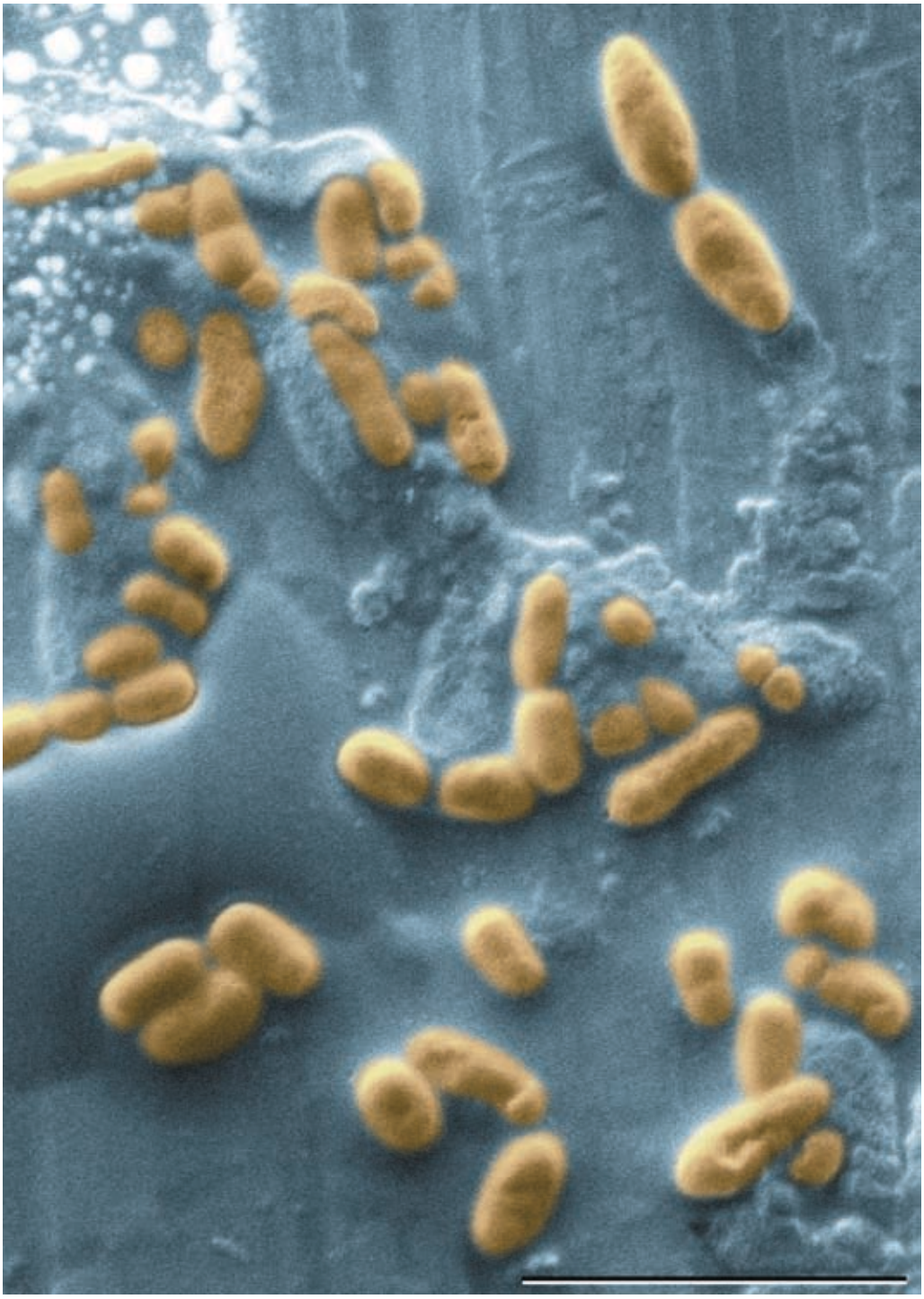


Fig. 12. Bacteria on a working stainless steel surface were replicated using a cellulose acetate strip wetted with acetone. The softened strip was pressed onto the steel surface and then allowed to dry. Then it was peeled off, trimmed, mounted on an SEM stub, and coated with gold. "Inversion" of the micrograph in Adobe Photoshop software changed concave shapes into convex ones. Bar: 5 µm.

replicas (imprints) show the bacteria as concave depressions in the strips. Reversing the grayscale mode converts the depressions into convex structures and produces more familiar images.

Scientific papers are regularly illustrated with original grayscale SEM micrographs.

Stereo images may be obtained to emphasise spatial distribution of bacteria in solid specimens and to enhance the impression of the cells as three-dimensional (3-D) structures. The stereo images are pairs of the same areas viewed at two different angles, the difference between them usually being 6° to 12°. When viewed through a stereoscope, such stereoscopic pairs give an impression of a 3-D object. In another approach, they may be combined into a single frame, whereby one micrograph would be tinted red and the other green, cyan, or blue. Using free software available on the Internet (www.stereoeye.jp), two grayscale micrographs may be combined into an "anaglyph" which would then be viewed using a pair of glasses with the left glass being red and the right glass being green, cyan, or blue. The resulting impression is of a single 3-D object (Figure 13). In most microscopes, the stage is tilted along the horizontal axis which means that before assembling the stereogram or anaglyph (www.magma.ca/~pavel/science/Anaglyph.htm) the micrographs need to be turned 90° clockwise.

Colouring micrographs

Scientific papers are regularly illustrated with original grayscale SEM micrographs. Popular science articles (Sachs 2008) in various magazines and on the Internet, however, show images of bacteria in vivid colours. Such colours differ from the natural colours of the colonies and are called "false colours". They appeal both to magazine art directors and the readers. Educational literature including text books is another good reason to colour micrographs, where colours contribute to a better understanding of difficult subjects.

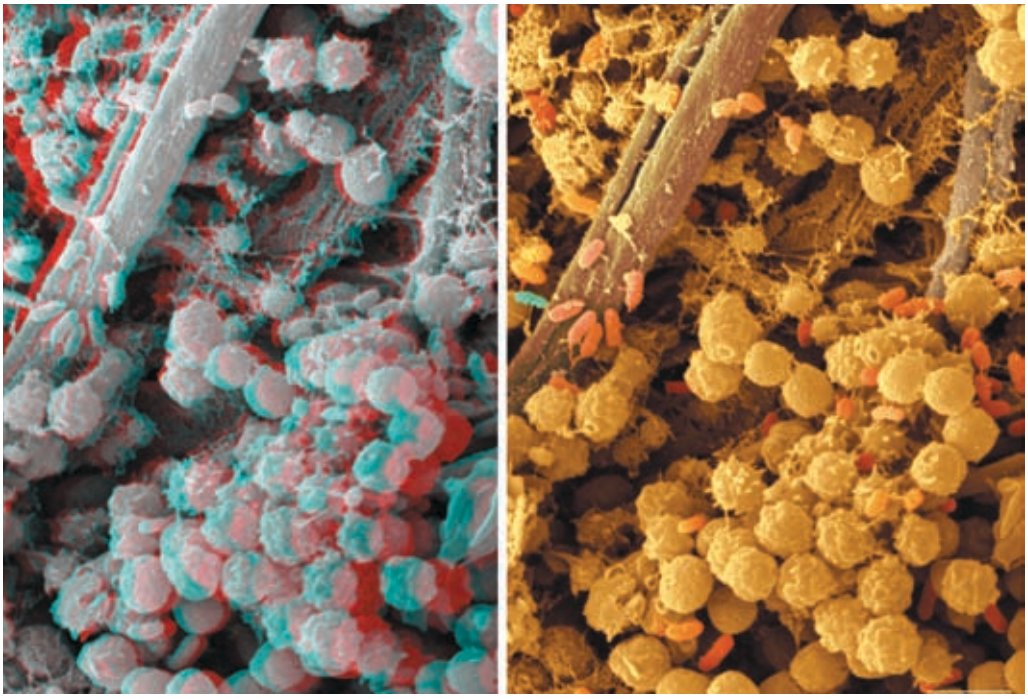


Fig. 13. A pair of micrographs showing the same area on a composted leaf. The image at right is a false colour micrograph and the image at left is a red-and-cyan anaglyph of the same area. When observed through a red-and-cyan pair of glasses, fungal spores in the lower half of the left image raise from the image plane. Bar: 2 µm.

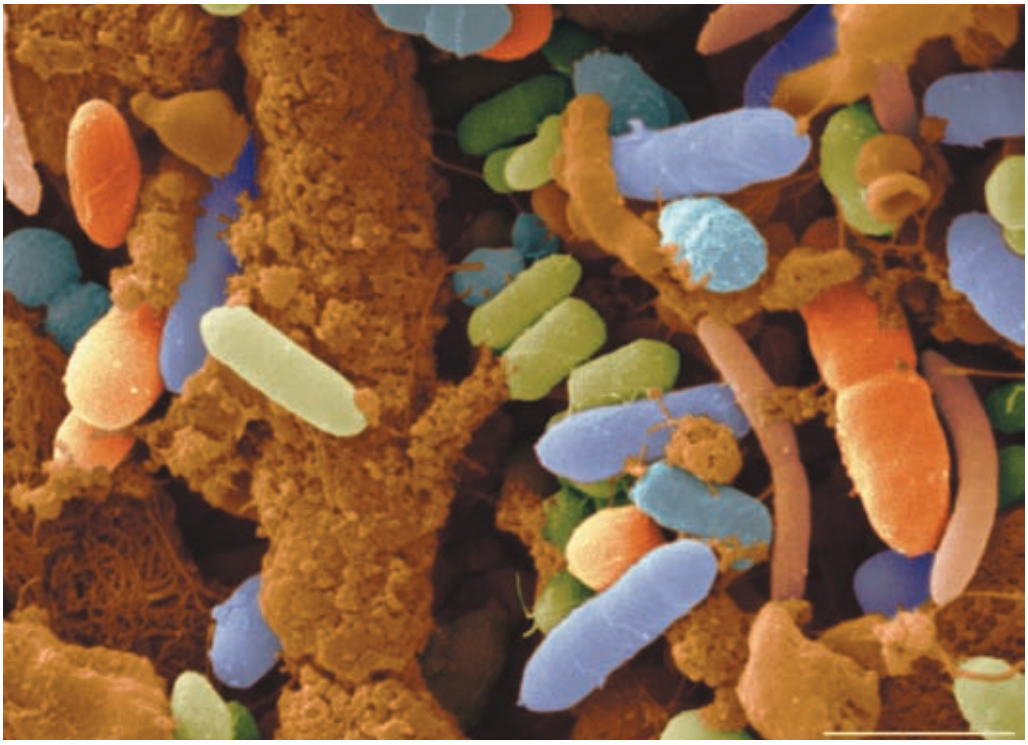


Fig. 14. A micrograph of various species of bacteria in human feces. Bacteria of similar shapes were selected and copied into separate layers in Adobe Photoshop PSD format. Each layer was assigned a specific colour different from other layers. The image was then flattened and the combined colours were further adjusted. Bar: 2 µm.

Micrographs in false colours are available online for illustration purposes from specialized microscopists, such as:

- www.denniskunkel.com
- www.scharfphoto.com
- www.microtechnics.com
- http://web.mac.com/camazine

Or from various scientifically oriented photobanks, such as:
- www.sciencesource.com
- www.cmsp.com
- www.mediscan.co.uk
- www.visualsunlimited.com
- www.sciencephoto.com/index.html

Microscopists with access to Adobe Photoshop, Corel Photo-Paint or other quality graphic software and a pointing device such as a mouse or, better, a graphic tablet and a pen, may colour their own micrographs using one of two different procedures which are, however, only two most common examples of colouring images.

(1) The micrograph is converted into the RGB Colour Mode and further converted into the "Indexed Colour Mode". This step reduces the total number of shades of gray to 256 which may be manually assigned desired colours. The image is then converted back into the RGB Colour Mode and its colours, contrast, and brightness are further adjusted. This procedure is useful for simple images and may be combined with the following procedure for complex images.

(2) A micrograph showing a variety of subjects (various bacteria and/or other subjects such as plant cells, and milk proteins, etc) is coloured in the RGB Colour Mode. Structures to be assigned similar colour (e.g., the blue bacteria in Figure 14) are manually selected using a mouse or a digitizing pen and a graphic tablet and are assigned the particular colours. This selected image in colour is made into a separate layer. Other subjects (bacteria) to be assigned a different colour are selected from the

basic image (background) and processed in a similar way. Finally, all layers are merged and the flattened image is saved. There are also other techniques of rendering raster images. Colouring complex micrographs is a long and tedious process.

Conclusions

Scanning electron microscopy renders bacteria as three-dimensional objects in various environments close to their natural appearance provided that the nature of the specimens is rigorously respected. Using a set of up to 6 small specimens, all necessary preparatory steps may be accomplished within a single working day and microscopy may proceed on the following day. Multiple specimen holders are particularly advantageous with bacterial suspensions captured on bacterial filters. Similar to SEM of any subjects, it is strongly recommended that other electron microscopy techniques, particularly TEM, be also used to characterise the bacteria under study in greater detail. Several suggestions on these topics are available on-line, e.g., at www.magma.ca/~scimat/foodmicr.htm.

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Following the invasion of Czechoslovakia in 1968, Miloš was hired by then Agriculture Canada to develop wieners from skimmed milk powder. To better understand the transformation of milk proteins, he learned to carry out most electron microscopy techniques thanks to a unique Electron Microscopy Lab system where scientists and technicians of various disciplines interacted and learned one from each other with the assistance from Dr. G. H. Haggis.

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